Effects of dental resin metabolites on estrogenic activity *in vitro*

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Three monomers (Bis-GMA, UDMA, and TEGDMA) and five polymerization initiators (CQ, BPO, DMPT, DMAEMA, and ATU) commonly used in dental composite resins were tested for estrogenic activity using a reporter gene assay (yeast two-hybrid system) *in vitro*, and compared with bisphenol-A (BPA). Estrogenic activity was indicated by agonist and antagonist activity, with (+S9) and without (−S9) metabolic activation using rat liver cells.

No estrogenic agonist activity was seen for each monomer and polymerization initiator in either the −S9 and +S9 tests in the concentration ranges examined in this study. On the other hand, estrogen antagonist activity was found with BPO and DMPT. BPO showed antagonist activity at a concentration of ~1800 nM with the −S9 test, but not with the +S9 test. With DMPT, antagonist activity was not seen with the −S9 test, but it was seen at a concentration of ~610 nM using the +S9 test. With BPA, the +S9 test indicated antagonist activity at a concentration of ~780 nM. The estrogen antagonist activities of DMPT and BPA appeared to be similar. CQ, DMAEMA, ATU, and the three monomers did not show antagonist activity as demonstrated by the −S9 or +S9 tests within the concentration range tested in this study.
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1. Introduction

Synthetic chemicals that act as endocrine disruptors (EDs) have raised concerns in the research world [1, 2]. EDs are usually ingested orally. The consequences of elevated ED concentrations in the mouse can be very serious. As a result, the safety of many materials used in dentistry, and their potential hazards for living organisms, have been the subject of considerable research effort [3–6].

In dental resins, polymerization initiators generate radicals that initiate polymerization by attacking monomer double bonds. However, polymerization is incomplete, and residual monomers, polymerization initiators, and contaminants leach into the saliva [7, 8]. BPA, which is the raw form of the Bis-GMA monomer, may leach from dental resins from remaining Bis-GMA monomer or the breakdown product of Bis-GMA [9]. This study investigated the estrogenic activity of some components of the most commonly used dental resins. These were three monomers (Bis-GMA, UDMA, and TEGDMA) and five polymerization initiators (CQ, BPO, DMPT, DMAEMA, and ATU).

Many methods are available for testing estrogenic activity [10–13]. Of these, *in vitro* screening tests are simple and useful tools for identifying suspected EDs. Such tests include the MCF-7 cell proliferation assay (E-Screen), receptor binding assays, and reporter gene assays using cultured cells and yeast cells [5, 14–17].

This study used an *in vitro* reporter gene assay using yeast cells (a yeast two-hybrid assay). This system is based on the ligand-dependent interaction of two proteins (a hormone receptor and a coactivator), and hormonal activity is detected by β-galactosidase activity. Estrogenic activity was indicated by agonist and antagonist activity with (+S9) or without (−S9) metabolic activation. The antagonist test used the Microtox test to determine whether the inhibition of β-galactosidase activity depended on the effects of chemicals on the receptor or on yeast toxicity.

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The estrogenic activity of BPA was compared with that of the monomers and polymerization initiators.

2. Materials and methods

2.1. Test chemicals

The monomers examined were 2,2-bis[4-(3-methacriloxy-2-hydroxypropoxy) phenyl] propane (Bis-GMA, Shin-Nakamura Chemical Co., Wakayama, Japan), dimethacryloxyethyl-2,2,4-trimethylhexamethylene diurathane (UDMA, Shin-Nakamura Chemical Co., Wakayama, Japan), and triethylene glycol dimethacrylate (TEGDMA, Tokyo Kasei Co., Tokyo, Japan). The polymerization initiators studied were camphorquinone (CQ, Tokyo Kasei Co., Tokyo, Japan), benzoyl peroxide (BPO, Katayama Chemical Co., Osaka, Japan), dimethyl para toluidine (DMPT, Tokyo Kasei Co., Tokyo, Japan), 2-dimethylamino-ethyl-methacrylate (DMAEMA, Tokyo Kasei Co., Tokyo, Japan), and 1-allyl-2-thiourea (ATU, Aldrich Chemical Co., Tokyo, Japan), which were used without further purification. Bis-phenol-A (BPA, Kanto Chemical Co., Tokyo, Japan) was prepared for comparison with these chemicals (Fig. 1).

The chemicals were diluted in dimethyl sulfoxide (DMSO, Kanto Chemical Co., Tokyo, Japan) at the experimental concentrations.

2.2. Yeast two-hybrid assay

The yeast two-hybrid assay with the estrogen receptor ERα and the coactivator TIF2 was used to measure estrogenic activity, as first described by Nishikawa *et al.* [14–16]. Briefly, yeast transformants, which carry a β-galactosidase reporter gene, were pre-incubated overnight at 30 °C in a selective medium. The culture (120 μL) in each well of a 96-well microplate (SUMILON, Sumitomo Bakelite Co., Tokyo, Japan) was then mixed with a DMSO solution (1.2 μL) of the test chemical (− S9) and incubated for 4 h at 30 °C; it was metabolized with S9 mix (Oriental Yeast Co., Tokyo, Japan) for 1 h at 37 °C in the + S9 test. In the antagonist test, 17β-estradiol (b-E2) (Sigma Chemical Co., St. Louis, MO, USA) was added to each well, at a 600 pM concentration, before incubation.

The cell walls of yeast were digested enzymatically by incubation with 80 μL of decomposition enzyme (zymolyase-20T, SEIKAGAKU CO., Tokyo, Japan) mixed with a chemiluminescent reaction buffer (Aurora Gal-XE Kit, ICN Pharmaceutical Inc., Tokyo, Japan) at 37 °C for 1 h.

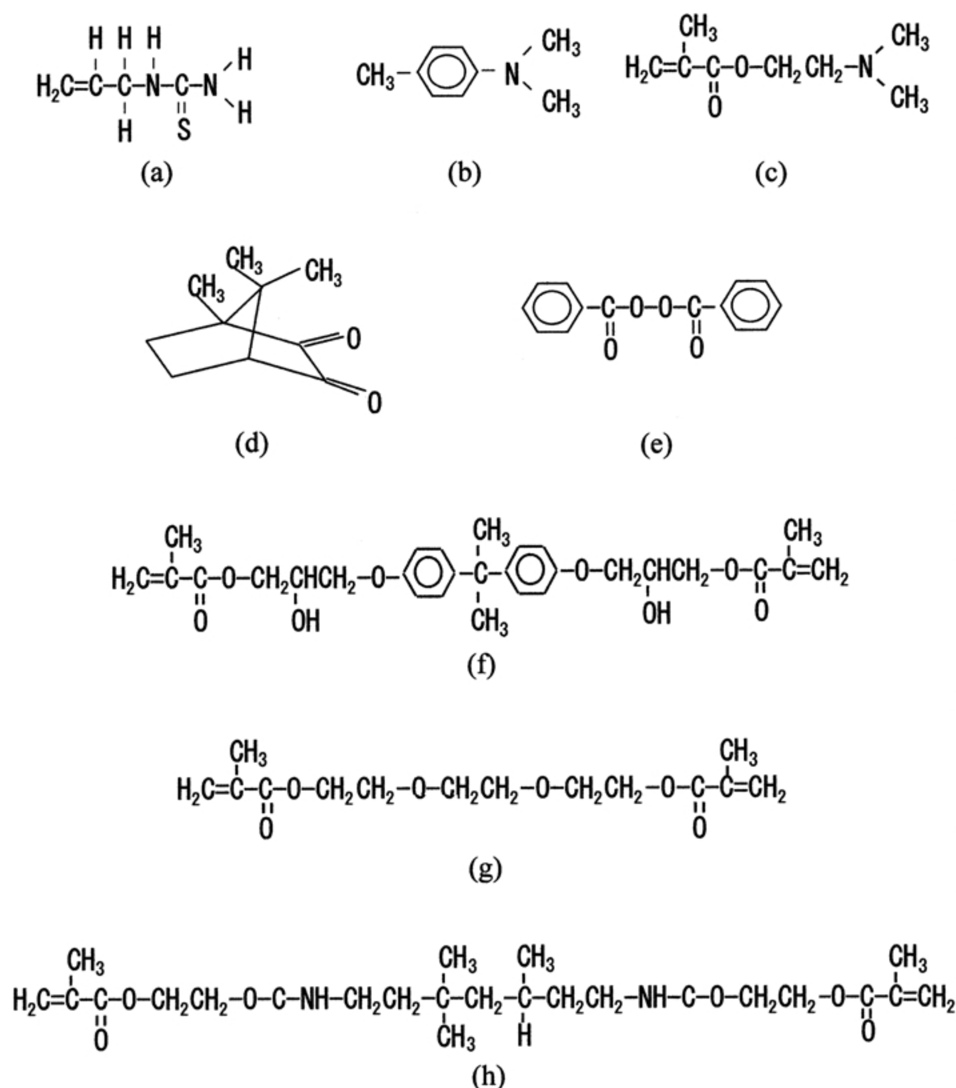

Figure 1 Molecule structure of the polymerization initiators and the monomers: (a) ATU; (b) DMPT; (c) DMAEMA; (d) CQ; (e) BPO; (f) Bis-GMA; (g) TEGDMA; and (h) UDMA.

The cultures in the 96-well microplates were mixed with 50 μL of a chemiluminescence promotion solution (Aurora Gal-XE Kit, ICN Pharmaceutical Inc., Tokyo, Japan), and the chemiluminescence of each well was measured using chemiluminescence assay instruments (AB2100, Luminescencer-JNR, ATTO Bio-Instrument, Tokyo, Japan) in order to estimate estrogenic activity.

The agonist activity was represented as the ratio of the chemiluminescent intensity of β-galactosidase to the baseline intensity, and was considered positive when it exceeded 10. The antagonist activity was represented as the inhibited rate (%) of 17β-estradiol (b-E2) activity and expressed as the 50% inhibition concentration (IC_{50}).

2.3. Microtox test

In the Microtox test, the yeast was incubated as in the estrogen activity test. The culture (120 μL) in each well of a 96-well microplate was mixed with a DMSO solution (1.2 μL) of test chemical or DMSO using the same concentration ranges as in the estrogen activity test.

The toxicities of chemicals were represented as the residual rate (%) of chemiluminescent intensity of β-galactosidase compared to the baseline level.

3. Results

No estrogenic agonist activity was seen for the monomers or polymerization initiators in either the − S9 or + S9 tests, while estrogenic antagonist occurred with BPO and DMPT. In the − S9 test, BPO showed antagonist activity at a concentration of ∼1800 nM(IC_{50}), but this activity was not seen in the + S9 test (Fig. 2). No antagonist activity was observed for DMPT in the −S9 test, while activity was observed in the + S9 test at a concentration of ∼610 nM(IC_{50}) (Fig. 3). The BPA control group showed antagonist activity in the + S9 assay at a concentration of ∼780 nM(IC_{50}) (Fig. 4). Toxicity of DMPT and BPO was not measured in the concentration range tested (less than 2000 nM). This means that these effects were due to estrogenic antagonist activity, and not to chemical toxicity. The other polymerization initiators (CQ, DMAEMA, and ATU) and monomers (Bis-GMA, UDMA, and TEGDMA) did not show toxicity in either the − S9 or + S9 tests for the concentration range studied (less than 2000 nM).

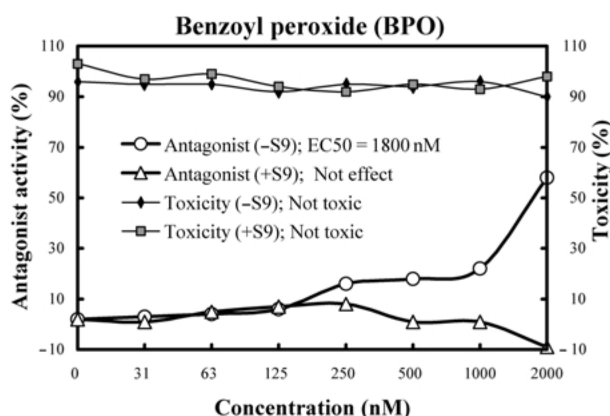

Figure 2 Dose-response curves for the agonist, antagonist, and Microtox toxicity tests, for BPO tested with − S9 and + S9. Antagonist activity for BPO occurred at a concentration of 1800 nM in the − S9 test.

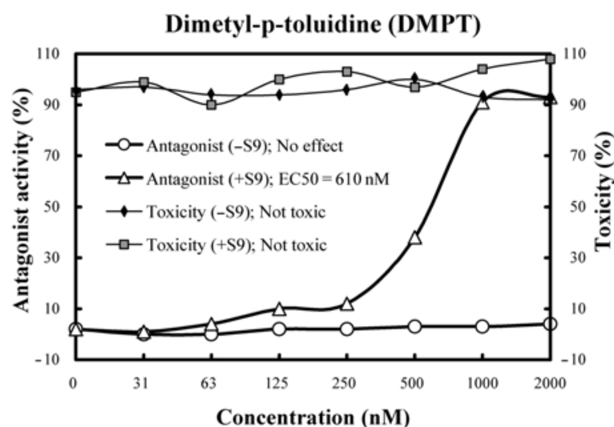

Figure 3 Dose-response curves for the agonist, antagonist, and Microtox toxicity tests, for DMPT tested with − S9 and + S9. Antagonist activity for DMPT occurred at a concentration of 610 nM in the + S9 test.

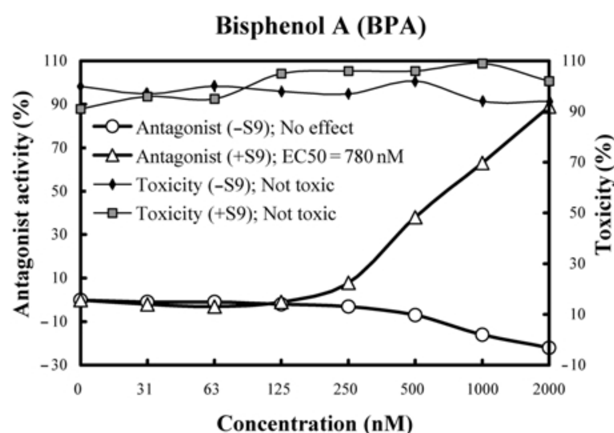

Figure 4 Dose-response curves for the agonist, antagonist, and Microtox toxicity tests, for BPA tested with − S9 and + S9. Antagonist activity for BPA occurred at a concentration of 780 nM in the + S9 test.

4. Discussion

Various materials are used in dentistry, and their harmful effects on the body result primarily in tooth pulp damage, contact dermatitis, and allergy [18–20]. The work of Olea *et al.* [21] on the detection of BPA in a Bis-GMA-based sealant has led to the recognition of the potential problems posed by endocrine disruptor chemicals in clinical dentistry [22, 23]. They tested the estrogenic activity of some resin-based composites used in dentistry and showed that contaminants from a Bis-GMA-based sealant altered the proliferative nature of cultured human breast cancer cells and the estrogenicity was due to bisphenol-A and bisphenol-A dimethacrylate, monomers found in the base paste of the dental sealant and identified by mass spectrometry [21].

We investigated whether this estrogenicity was caused by the original Bis-GMA using a yeast hybrid system *in vitro*. However, we did not see estrogen agonist activity in the − S9 or + S9 tests with Bis-GMA, UDMA, or

TEGDMA. The antagonist test had similar results. It is thought that the contaminants arise during the manufacture of the Bis-GMA-based sealant.

The polymerization initiators did not show estrogen agonist activity in either the − S9 or + S9 tests. However, antagonist activity of BPO was seen in the − S9 test at concentrations of ∼1800 nM, while it was lost with metabolic activation (+ S9). DMPT showed no estrogen antagonist activity in the −S9 test, but showed some effect in the + S9 test at a concentration of ∼610 nM. In the + S9 test, under the same test conditions, BPA showed antagonist activity at ∼780 nM; therefore, the estrogen antagonist activity of DMPT is similar to that of BPA.

Commercial dental resin usually contains about 0.2–1.0 mg DMPT per 100 mg monomer. An organic solvent (methanol) will reduce 0.5 mg of DMPT in 100 mg of dental resin to about 50 μg, over a 24-h period [24]. In the body, these diluted chemicals from the mouth enter the bloodstream via the intestinal tract and pass through the liver before traveling throughout the body. Therefore, great attention should be paid to chemicals that gain estrogenic activity after being metabolized in the liver (antagonist), such as DMPT. BPA showed estrogenic activity after metabolization; however, several studies have reported that no BPA is eluted from polymerized sealants [3, 25].

In this study, we determined that two polymerization initiators (BPO, DMPT) have estrogenic activity *in vitro*; however, this may not be sufficient to determine whether they are estrogenic. More data based on physiological and biochemical tests, and *in vivo* studies are needed.

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